Postcopulatory sexual selection generates speciation phenotypes in Drosophila

Manier, Mollie K ; Lüpold, Stefan ; Belote, John M ; Starmer, William T ; Berben, Kirstin S ; Ala-Honkola, Outi ; Collins, William F ; Pitnick, Scott

Abstract: BACKGROUND Identifying traits that reproductively isolate species, and the selective forces underlying their divergence, is a central goal of evolutionary biology and speciation research. There is growing recognition that postcopulatory sexual selection, which can drive rapid diversification of interacting ejaculate and female reproductive tract traits that mediate sperm competition, may be an engine of speciation. Conspecific sperm precedence (CSP) is a taxonomically widespread form of reproductive isolation, but the selective causes and divergent traits responsible for CSP are poorly understood. RESULTS To test the hypothesis that postcopulatory sexual selection can generate reproductive isolation, we expressed GFP or RFP in sperm heads of recently diverged sister species, Drosophila simulans and D. mauritiana, to enable detailed resolution of species-specific sperm precedence mechanisms. Between-species divergence in sperm competition traits and mechanisms prompted six a priori predictions regarding mechanisms of CSP and degree of cross asymmetry in reproductive isolation. We resolved four distinct mechanisms of CSP that were highly consistent with predictions. These comprise interactions between multiple sex-specific traits, including two independent mechanisms by which females exert sophisticated control over sperm fate to favor the conspecific male. CONCLUSIONS Our results confirm that reproductive isolation can quickly arise from diversifying (allopatric) postcopulatory sexual selection. This experimental approach to "speciation phenotypes" illustrates how knowledge of sperm precedence mechanisms can be used to predict the mechanisms and extent of reproductive isolation between populations and species.

DOI: https://doi.org/10.1016/j.cub.2013.07.086

Current Biology 23, 1853–1862, October 7, 2013 ©2013 Elsevier Ltd All rights reserved http://dx.doi.org/10.1016/j.cub.2013.07.086

Article

Postcopulatory Sexual Selection Generates Speciation Phenotypes in *Drosophila*

Mollie K. Manier,[1] Stefan Lüpold,[1] John M. Belote,[1] William T. Starmer,[1] Kirstin S. Berben,[1] Outi Ala-Honkola,[2] William F. Collins,[1] and Scott Pitnick[1,*]

[1]Department of Biology, Syracuse University, Syracuse, NY 13244, USA

[2]Department of Biological and Environmental Science, University of Jyväskylä, 40014 Jyväskylä, Finland

Summary

Background: Identifying traits that reproductively isolate species, and the selective forces underlying their divergence, is a central goal of evolutionary biology and speciation research. There is growing recognition that postcopulatory sexual selection, which can drive rapid diversification of interacting ejaculate and female reproductive tract traits that mediate sperm competition, may be an engine of speciation. Conspecific sperm precedence (CSP) is a taxonomically widespread form of reproductive isolation, but the selective causes and divergent traits responsible for CSP are poorly understood.

Results: To test the hypothesis that postcopulatory sexual selection can generate reproductive isolation, we expressed GFP or RFP in sperm heads of recently diverged sister species, *Drosophila simulans* and *D. mauritiana*, to enable detailed resolution of species-specific sperm precedence mechanisms. Between-species divergence in sperm competition traits and mechanisms prompted six a priori predictions regarding mechanisms of CSP and degree of cross asymmetry in reproductive isolation. We resolved four distinct mechanisms of CSP that were highly consistent with predictions. These comprise interactions between multiple sex-specific traits, including two independent mechanisms by which females exert sophisticated control over sperm fate to favor the conspecific male.

Conclusions: Our results confirm that reproductive isolation can quickly arise from diversifying (allopatric) postcopulatory sexual selection. This experimental approach to "speciation phenotypes" illustrates how knowledge of sperm precedence mechanisms can be used to predict the mechanisms and extent of reproductive isolation between populations and species.

Introduction

Evolutionary biology seeks to understand diversification. The origin of new species links microevolutionary processes driving divergence among populations with macroevolutionary processes driving cladogenesis, yet how new species form remains poorly understood [1]. The role of sexual selection has been the focus of vigorous investigation and contention [2–4]. Intuitively, because precopulatory sexual selection drives the rapid evolution of traits that determine the outcome of encounters between the sexes (e.g., male ornaments, courtship behavior, and female preferences), it is predicted to be a widespread agent of reproductive isolation [2–4]. Investigations of the role of sexual selection in speciation, both empirical and theoretical, have overwhelmingly addressed precopulatory rather than postcopulatory selection [1–3, 5]. This disparity may be attributable to the inherent difficulties of establishing the role of postcopulatory sexual selection in character divergence and reproductive isolation given the cryptic nature of ejaculate-ejaculate and ejaculate-female interactions [5, 6].

There is nevertheless growing recognition of the potential importance of postmating/prezygotic (PMPZ) reproductive isolation [2, 5], due to mounting evidence that (1) ejaculate-female interactions are complex [7, 8], (2) seminal, sperm, and female reproductive tract traits evolve rapidly [8] with interacting, sex-specific traits exhibiting patterns of correlated evolution [8, 9], and (3) conspecific sperm precedence (CSP) is a taxonomically widespread PMPZ isolating mechanism [5]. CSP is a specific form of gametic isolation in which noncompetitive hybrid matings produce near-normal numbers of offspring, but sperm competition between a conspecific and heterospecific male produces primarily conspecific offspring, irrespective of mating order. CSP has been documented in internal fertilizers, broadcast spawners, and plants (as conspecific pollen precedence; [5, 10]), and in some instances it is the only discernable form of reproductive isolation [11]. However, mechanisms underlying CSP in internal fertilizers remain poorly resolved due to observational limitations of the female reproductive tract, compounded by difficulties differentiating conspecific from heterospecific sperm. Successes in partially overcoming these obstacles are rare (e.g., [11–14]).

A recent analysis of the state of speciation research has recommended a shift toward (1) characterizing "speciation phenotypes" important in reproductive isolation, (2) identifying selective forces driving their divergence, (3) resolving their genetic basis, and (4) understanding the relationship between microevolutionary phenotypic evolution within species and macroevolutionary divergence between species [15]. Notably, some of the best examples of research programs applying this approach are with systems for which divergence is at least partially attributed to precopulatory sexual selection (e.g., [16, 17]). Here, we employ these recommendations while examining the relationship between trait divergence attributable to postcopulatory sexual selection within species and the mechanisms of reproductive isolation between species. Specifically, we ask whether knowledge of phenotypic divergence between sister species in postcopulatory sexually selected traits can predict patterns of reproductive isolation and the causal mechanisms underlying such isolation.

Drosophila simulans and *D. mauritiana* diverged an estimated 260,000 years ago [18] and exhibit CSP in double matings with *D. simulans* females [12, 19]. A previous investigation used spermless *D. simulans* males to track the fate of *D. mauritiana* sperm in double matings and found that *D. simulans* seminal fluid alone was sufficient to reduce the number of first-male *D. mauritiana* sperm in storage and to interfere with use of second-male *D. mauritiana* sperm for fertilizations [12]. We have recently used transgenic lines of each

*Correspondence: sspitnic@syr.edu

Table 1. Six A Priori Predictions and Their Justification Regarding Causal Mechanisms and Patterns of Postmating, Prezygotic Reproductive Isolation between *D. simulans* and *D. mauritiana*

Prediction 1	To the extent that divergence attributable to postcopulatory sexual selection generates reproductive isolation, mechanisms underlying CSP will include some of the four critical reproductive events: sperm transfer, sperm displacement, sperm ejection, and fertilization bias [20, 24].
Prediction 2	Both *D. simulans* and *D. mauritiana* males have been shown to significantly tailor the number of sperm transferred based on aspect of female quality (i.e., mating status) [20]. We predicted that males of both species would transfer fewer sperm to heterospecific females, hence contributing to CSP but not to any asymmetry in CSP between female backgrounds.
Prediction 3	Due to their greater length, *D. simulans* sperm will be superior at displacing and resisting displacement by *D. mauritiana* sperm within *D. simulans* females, which have a longer seminal receptacle (SR) [20]. In contrast, *D. mauritiana* sperm will not have a displacement advantage over *D. simulans* sperm within *D. mauritiana* females.
Prediction 4	Sperm ejection is significantly mediated by females in *D. melanogaster* [22, 23] and there is a difference between species in the time of ejection after remating (*D. simulans*, 130 ± 6 min; *D. mauritiana*, 101 ± 6 min) [20]. However, because we have no knowledge of putative male×female influences on ejection time, we predict no asymmetry in ejection time based on female species identity. If females use sperm ejection to discriminate against lower "quality" males, then both *D. simulans* and *D. mauritiana* females will eject heterospecific ejaculates more rapidly than conspecific ejaculates.
Prediction 5	Regarding fertilization bias, *D. simulans* females exhibit the potential to exert sophisticated control over the fertilization set to bias fertilization in favor of conspecific sperm, whereas *D. mauritiana* females do not [21].
Prediction 6	Due to predictions 3 and 5, we predict a stronger pattern of CSP when conspecific and heterospecific sperm compete within *D. simulans* females than when competition occurs within *D. mauritiana* females.

species, expressing GFP or RFP in sperm heads (Movies S1, S2, and S3 available online), to resolve their respective, species-specific sperm precedence mechanisms. Those investigations revealed a shared motif in the processes that determine the pattern of sperm precedence, yet substantive quantitative and qualitative evolutionary differentiation in traits participating in these processes [20, 21]. Additionally, investigations using isogenic lines of the closely related *D. melanogaster* have established relationships between within-population genetic variation in these same traits and competitive fertilization success [22, 23]. Our knowledge of divergence between *D. simulans* and *D. mauritiana* in sperm precedence mechanisms led to the prediction that CSP should be weaker for successful hybrid inseminations of *D. mauritiana* than of *D. simulans* females, in addition to specific predicted mechanisms underlying CSP (Table 1).

Here, we test the predicted isolation pattern and speciation phenotypes (Table 1) by quantifying patterns of sperm transfer, storage, displacement, ejection, and use for fertilizations when *D. simulans* and *D. mauritiana* males compete against one another within both female backgrounds in order to resolve mechanisms of CSP in this system. Whereas divergence in CSP traits can theoretically proceed by selection against hybridization (i.e., reinforcement [25]), *D. mauritiana* arose from a *D. simulans*-like ancestor in allopatry, and without secondary contact [26]. Hence, by evaluating the extent to which mechanisms of CSP are predicted by among-species divergence in sperm precedence traits and mechanisms, we provide a strict assessment of the contribution of divergent postcopulatory sexual selection, per se, to reproductive isolation using a model system approach. Because *D. mauritiana* females exhibit strong premating discrimination against *D. simulans* males [19, 26], the main focus of this study is on events during CSP in *D. simulans* females. However, we were able to perform limited tests of certain critical hypotheses in *D. mauritiana* females and include those results.

Results

CSP in *Drosophila simulans* Females

Our results confirm gametic isolation in the form of CSP when hetero- and conspecific sperm compete within *D. simulans* females. Specifically, female *D. simulans* (S) produced as many offspring after a single mating with a heterospecific (*D. mauritiana*, M) male as with a conspecific male (S×M, mean ± SE, 57.9 ± 7.81, n = 46; S×S, 62.4 ± 7.16, n = 27; $t_{67.7} = -0.22$, p = 0.83; Figure 1) and with no difference in hatch rate (S×S, 0.78 ± 0.05, n = 26; S×M, 0.73 ± 0.06, n = 36; $t_{54.9} = -1.19$, p = 0.24). After double matings, however, conspecific males sired the majority of progeny irrespective of mating order (P_2: S×MS, 0.84 ± 0.05, n = 33; S×SM, 0.21 ± 0.05, n = 54; Table 2).

The first mechanism of CSP confirmed a previous report [12] of reduced insemination success in rematings with heterospecific males. Intraspecific insemination during remating within M×MM or S×SS crosses were nearly 100% successful, and those within the S×MS cross were 92% successful. However, the success rate dropped to 50% and 30% for S×SM and S×MM crosses, respectively (Figure 2A and Table 2). Further, when we considered only those pairings resulting in successful insemination, there was a significant interaction between first- and second-male species identity on the number of sperm transferred ($F = 35.3$, p < 0.0001; n = 175). This interaction is attributable to two patterns: (1) *D. mauritiana* males transferred 16% fewer sperm when preceded by another *D. mauritiana* male (S×MM) rather than by a *D. simulans* male (S×SM) and (2) *D. simulans* males transferred 48% fewer sperm when preceded by a *D. mauritiana* male (S×MS) rather than by another *D. simulans* male (S×SS; Figure 2B and Table 2).

The second mechanism of CSP involves sperm displacement. Despite transferring only half the sperm number of the S×SS cross, *D. simulans* males in the S×MS treatment displaced 75% of their rivals' sperm in storage, a significant increase over the mean S×SS displacement rate of 38%. In sharp contrast, *D. mauritiana* males in the S×SM treatment displaced only 12% of the *D. simulans* sperm residing in storage, an approximately 2-fold decrease from the 23% displacement of the S×MM cross and a 4-fold decrease from the 48% displacement of the M×MM cross (Figure 2C and Table 2). In other words, *D. simulans* sperm are better able to displace and resist displacement by *D. mauritiana* sperm within *D. simulans* females (Figure 3).

The decreased ability of *D. mauritiana* sperm to compete against *D. simulans* sperm within *D. simulans* females was further compounded by the third identified mechanism of CSP: significantly faster sperm ejection by females of heterospecific ejaculates (Figures 2D and 3 and Table 2). As

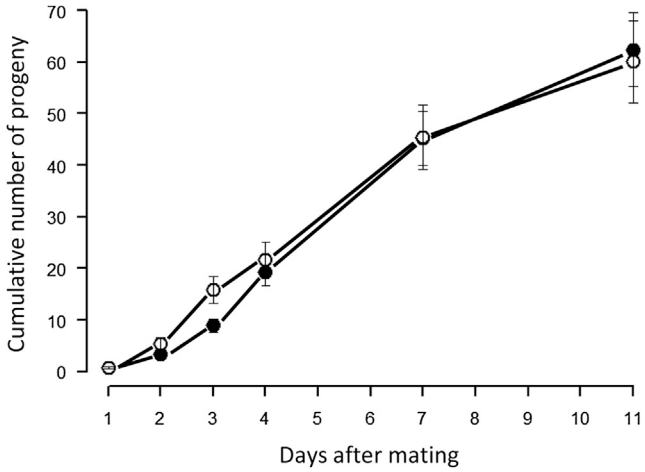

Figure 1. Progeny Production after Single Matings for *D. simulans* and *D. mauritiana*

The number of progeny produced after a single insemination does not differ between females mated to a conspecific male and those mated to a heterospecific male. The total cumulative number of progeny (means ± SE) produced over 11 days in S×S (black) and S×M (white) crosses is shown.

second-male sperm move from the bursa into the storage organs, some stored sperm (presumably both first and second male), are displaced back into the bursa (increasingly so as the organs approach their storage capacity; Figure 3) [22, 24]. This process will eventually result in equilibrium of first- and second-male sperm proportions between the storage organs and the bursa. We have suggested that female ejection of sperm from the bursa prior to this equilibrium being reached is most likely to influence paternity success [20]. No ejections occurred before this time point (i.e., 60 min after mating) in the S×SS cross [20], whereas 11 of 15 females (73%) ejected by this time in the S×SM cross. In addition, females ejected heterospecific sperm especially rapidly when previously mated to a conspecific male, often immediately after the male dismounted, and females retained a conspecific ejaculate for an especially long time when previously mated to a heterospecific male (S×MS ejection time 577% longer on average than the S×SM cross; Figures 2D and 3 and Table 2). Further, we anecdotally observed some females in heterospecific rematings (S×SM or S×MM), but never in conspecific rematings, experiencing difficulty ejecting the sperm mass in a process that can last 1 hr (Movie S4), possibly due to biochemical ejaculate-female incompatibility.

Sperm remaining postejection within the storage organs potentially constitute the "fertilization set," or the population of sperm competing to fertilize eggs [20]. We developed an analytical model [21] that estimates bias between storage organ types (i.e., the paired spermathecae and the seminal receptacle [SR]) in providing sperm for fertilization (z) and hence more specifically identifies the fertilization set, in addition to any first- or second-male fertilization biases (i.e., sperm use disproportionate to their representation) in the spermathecae (x) and SR (y) (Figures 4A and 4B). We applied this model to intraspecific sperm competition in *D. melanogaster*, *D. simulans*, and *D. mauritiana* [21]. In contrast to *D. melanogaster*, which primarily uses sperm from the SR for fertilization [20–24], sperm from both organ types contribute equally to the fertilization set in *D. simulans* (Figure 4C) and *D. mauritiana* [21]. Whereas no fertilization bias was observed in either organ in *D. melanogaster* and *D. mauritiana*, female *D. simulans* uniquely exhibited second-male sperm bias in the spermathecae and first-male sperm bias in the SR (Figure 4C).

Here, we applied the same analytical model to the CSP cross data for *D. simulans* females. The previously observed [21] pattern of opposing fertilization bias between the SR and spermathecae was found to be consistent across all treatments (compare bars x and y among Figures 4C–4E), suggesting a strictly female-mediated phenomenon. Here we additionally provide evidence that females exploit this mechanism to favor the conspecific male by adjusting the extent to which sperm for fertilization are released from the SR or spermathecae, depending upon mating order, thus providing a fourth, highly sophisticated mechanism of CSP. There was a significant difference between the reciprocal CSP treatments in the sources of sperm for fertilization ($t_{50} = 2.8$, p = 0.0072; compare bar z in Figures 4D and 4E). Specifically, females in the S×MS cross significantly favored the spermathecae, which has a second-male sperm bias, thus favoring the conspecific male (Figure 4D; $z = 0.28 \pm 0.05$; $t_{24} = 4.07$, p = 0.0006). In contrast, sperm use by females in the S×SM cross trended toward using more sperm from the SR, which has a first-male sperm bias, thus also favoring the conspecific male ($z = 0.71 \pm 0.14$), though it was not significantly different from $z = 0.5$ (Figure 4E; $t_{26} = -1.53$, p = 0.12).

Finally, we examined how cross-species interactions between rival sperm differ from within-species interactions with regard to sperm velocity in the SR. In *D. melanogaster*, experiments using isogenic lines with fixed ejaculate quality differences have revealed that rival sperm significantly influence one another's velocity, and slower sperm are better at displacing and resisting displacement by rival sperm [22]. Further, in *D. melanogaster*, *D. simulans*, and *D. mauritiana*, sperm velocity increases with time spent in storage, but this presumptively detrimental change is partially "rescued" by female remating and the presence of a rival ejaculate [20] (Figures 5A and 5B). In contrast to these patterns, we found here that *D. mauritiana* sperm velocity did not change over time within a *D. simulans* SR or in response to female remating, irrespective of the species identity of the second male (S×MM, $F_{3,84} = 0.20$, p = 0.90; S×MS, $F_{3,76} = 0.58$, p = 0.63; Figures 5C and 5D). In addition, *D. simulans* sperm velocity was not rescued by heterospecific insemination in S×SM matings (Figure 5E). These differences in sperm performance dynamics between intraspecific and heterospecific inseminations are likely to be symptomatic of trait divergence disrupting complex interactions between competing ejaculates and females [28].

CSP in *Drosophila mauritiana* Females

As expected due to strong premating isolation between *D. mauritiana* females and *D. simulans* males [19, 26], hybrid mating rates with *D. mauritiana* females were very low (5% for M×S and 1.6% for M×MS). But note that even among conspecific double matings (M×MM), only 40.0% of pairs copulated (Table 3). Only a single *D. mauritiana* female produced progeny after insemination by both a conspecific and heterospecific male (i.e., M×MS cross). This female produced six progeny with $P_2 = 0.67$.

In contrast to hybrid crosses with *D. simulans* females (Table 2), almost all hybrid matings that did occur with *D. mauritiana* females resulted in successful sperm transfer (100% and 93% for M×S and M×MS, respectively; Table 3). The average number of sperm transferred by *D. simulans* males to virgin

Table 2. Sperm Transfer, Storage, and Fate Differ Depending on Whether Conspecific or Heterospecific Ejaculates Are Competing and Interacting with *Drosophila simulans* Females

	S×S[a]	S×SS[a]	M×MM[a]	S×M	S×MM	S×SM	S×MS
Mating rate	0.94	0.84	0.40	0.80	0.85	0.88	0.74
	602	474	589	580	224	238	212
Proportion of successful matings	0.98	0.99	0.98	0.45	0.30	0.50	0.92
	53	139	109	31	13	44	24
Copulation duration (min)	21.4 ± 0.26	25.3 ± 0.44	23.7 ± 0.69	11.3 ± 0.26	11.7 ± 0.60	10.8 ± 0.50	26.4 ± 0.73
	574	204	141	333	141	171	106
	A	BC	C	D	D	D	B
Number of sperm transferred	1340 ± 96.7	2796 ± 152	2348 ± 93.9	1390 ± 109	1523 ± 132	1439 ± 134	1446 ± 165
	21	36	61	7	13	22	23
	A	B	B	A	A	A	A
Sperm stored (SR)	335 ± 16.8	329 ± 13.4	315 ± 11.9	209 ± 39.4	353 ± 22.0	177 ± 25.8	284 ± 16.2
	38	113	72	27	32	23	37
	A	A	A	B	A	BC	AB
Sperm stored (spermathecae)	200 ± 15.1	264 ± 12.6	237 ± 12.5	37.8 ± 21.9	144 ± 54.5	79.4 ± 16.6	165 ± 14.1
	31	88	61	9	7	23	19
	B	A	AB	C	BC	BC	AB
Sperm stored (total)	516 ± 25.7	587 ± 20.3	545 ± 21.4	204 ± 75.5	443 ± 81.2	284 ± 43.0	425 ± 33.6
	31	86	61	9	7	19	19
	AB	A	AB	C	BC	C	BC
Sperm stored (second male only)	NA	510 ± 20.0	425 ± 23.0	NA	346 ± 88.4	168 ± 38.5	400 ± 37.5
		90	61		7	23	19
		A	A		AB	B	A
Residual sperm at remating	NA	134 ± 10.0	252 ± 8.8	NA	222 ± 20.0	198 ± 15.3	161 ± 19.2
		180	160		53	108	39
		A	C		BC	B	AB
Ejection time (min ASM)	183 ± 5.2	116 ± 10.7	117 ± 4.1	111 ± 9.3	84.5 ± 16.4	26.7 ± 11.9	154 ± 15.6
	43	13	51	28	26	14	17
	A	BC	BC	BC	C	D	AB
Proportion of sperm displaced	NA	0.38 ± 0.04	0.48 ± 0.04	NA	0.23 ± 0.07	0.12 ± 0.04	0.75 ± 0.06
		77	61		10	29	23
		A	B		AB	B	C
P_2	NA	0.80 ± 0.03	0.88 ± 0.02	NA	NA	0.21 ± 0.05	0.84 ± 0.05
		63	79			54	33
		A	A			B	A
S2 (SR)	NA	0.82 ± 0.02	0.88 ± 0.02	NA	NA	0.19 ± 0.04	0.88 ± 0.04
		63	77			59	33
		A	A			B	A
S2 (spermathecae)	NA	0.77 ± 0.05	0.88 ± 0.02	NA	NA	0.15 ± 0.04	0.83 ± 0.05
		63	61			58	33
		A	A			B	A
x	NA	0.83 ± 0.05	0.46 ± 0.13	NA	NA	0.83 ± 0.12	0.87 ± 0.07
y	NA	0.37 ± 0.05	0.58 ± 0.08	NA	NA	0.24 ± 0.04	0.05 ± 0.03
z	NA	0.44 ± 0.08	0.38 ± 0.08	NA	NA	0.71 ± 0.14	0.28 ± 0.05
		65	80			25	27
Sperm velocity of first male (μm/s)	41.1 ± 6.0	46.1 ± 4.3	40.1 ± 2.4	56.3 ± 7.4	58.5 ± 4.6	80.0 ± 6.2	48.5 ± 3.6
	13	19	16	19	26	24	19
	A	AB	AB	AB	B	C	AB

Trait means ± SEM, sample size, and post hoc comparisons by Tukey's studentized range test ($\alpha = 0.05$; significant differences among crosses indicated by different letters) for first (S×S) and second (S×SS) matings of *D. simulans*, second matings of *D. mauritiana* (M×MM), and first (S×M) and second (S×MM, S×SM, and S×MS) hybrid matings.
[a]Data are from [20].

D. mauritiana females (M×S, 2758 ± 242, n = 6) was higher than for either type of intraspecific virgin insemination (S×S, 1,340 ± 97, n = 21; M×M, 1532 ± 103, n = 22; $F_{2,46} = 20.3$, $p < 0.0001$). Mean number of sperm transferred in the M×SM cross was comparable to that in hybrid crosses with *D. simulans* females (S×MM, S×SM, and S×MS; Table 2), but the distribution of ejaculate size in this cross was bimodal, with most males transferring over 1,000 sperm (average 2,123 ± 261.5, n = 6) and a few transferring less than 150 (average 83 ± 30, n = 3). Unexpectedly, *D. mauritiana* males mating with a conspecific female previously inseminated by a *D. simulans* male transferred far fewer sperm (276 ± 152, n = 4).

In contrast to patterns of sperm displacement observed for crosses with *D. simulans* females, we found no evidence of a competitive advantage of *D. mauritiana* sperm within *D. mauritiana* females. In fact, heterospecific *D. simulans* males were as effective as *D. mauritiana* males at displacing resident *D. mauritiana* sperm (M×MS, 56% ± 17%, n = 9; M×MM, 48% ± 0.04%, n = 57; Table 3 and Figure 2), despite having transferred approximately half as many sperm (see above; Table 3). A relatively small proportion of resident *D. simulans* sperm were displaced upon conspecific remating by females with *D. mauritiana* males (M×SM, 9% ± 6%, n = 4), but because so few sperm were transferred in these rematings (see above; Table 3), we caution against using these data to infer the relative resistance of heterospecific sperm to displacement within *D. mauritiana* females.

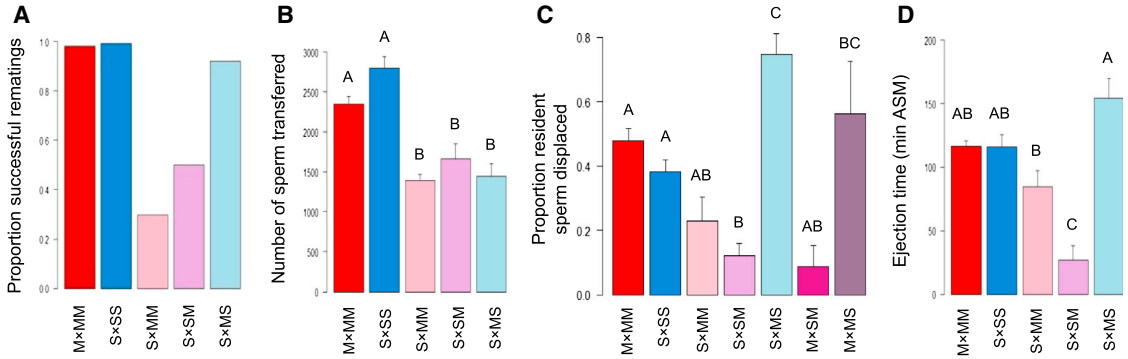

Figure 2. Multiple Mechanisms of CSP between *D. simulans* and *D. mauritiana*

Bars represent means ± SEM, and letters above bars indicate significant differences ($p < 0.05$) among all treatments shown. M, *D. mauritiana*; S, *D. simulans*; e.g., S×SM indicates a *D. simulans* female, first mated to *D. simulans* male, then remated to *D. mauritiana* male. All data refer to the female's second mating.
(A) Proportion of matings resulting in successful transfer of sperm.
(B) Number of sperm transferred per mating.
(C) Proportion of resident, first-male sperm displaced from the storage organs into the bursa.
(D) Time elapsed after the start of mating (ASM) until ejection of excess and displaced sperm by the female.
Error bars represent means ± 1 SEM, and letters above bars indicate significant differences ($p < 0.05$) among all treatments shown.

Patterns of sperm ejection in hybrid crosses with *D. mauritiana* females also differed dramatically from those with *D. simulans* females. With hybrid inseminations of *D. mauritiana*, ejection never occurred within 5 hr after mating in either single or double matings in either mating order (n = 20; Table 3). In other words, females remating to conspecific males have abnormal ejection behavior if they previously mated with a *D. simulans* male. In one case, the female still had not ejected the sperm mass (nor laid any eggs) by 72 hr after mating. These results suggest that the ejaculate plays an important role in ejection behavior and that the first male's ejaculate may influence ejection of the second male's ejaculate.

Discussion

Recent investigations of within-species mechanisms of sperm precedence in *D. melanogaster*, *D. simulans*, and *D. mauritiana* and have revealed the opportunity for postcopulatory sexual selection to occur during any of four reproductive events: (1) sperm transfer during copulation, (2) displacement of "resident" sperm from the storage organs by incoming sperm from the most recent mate, (3) female ejection of sperm from her reproductive tract, and (4) sperm use for fertilization [20–24]. Regarding prediction 1 (Table 1), we found that all four of these key reproductive events contribute to CSP between *D. simulans* and *D. mauritiana*. For heterospecific sperm competition within *D. simulans* females, CSP was attributable to a combination of (1) fewer copulations resulting in successful insemination when mating with *D. mauritiana* than with *D. simulans* males; (2) superior ability of *D. simulans* sperm to displace, and resist displacement by, *D. mauritiana* sperm; (3) faster ejection of *D. mauritiana* ejaculates; and (4) a shift in sperm use to the SR or spermathecae depending on male mating order such that fertilization was biased in favor of conspecific sperm. All four mechanisms involve interactions between multiple male and female traits, suggesting that reproductive isolation arises when interacting trait complexes that have coevolved and diverged in allopatry become disrupted in hybrid matings. These isolating mechanisms (i.e., speciation phenotypes) support the hypothesis that postcopulatory sexual selection can be a powerful agent of reproductive isolation.

We examined two aspects of insemination: whether copulation resulted in successful sperm transfer and the number of sperm transferred. Although no a priori prediction addressed the first aspect, it turned out to be a significant contributor to both CSP and its asymmetry between female backgrounds. Heterospecific matings with *D. simulans* females were significantly shorter in duration and less likely to result in successful insemination than were conspecific matings, whereas these variables did not differ between heterospecific and conspecific matings with *D. mauritiana* females. We have no knowledge of the mechanisms or the respective contribution of the sexes to these results. This difference in insemination success between cross types further magnifies prediction 6.

Regarding the number of sperm transferred for those copulations that did result in successful insemination, prediction 2 was not supported. Contrary to the pattern predicted, for single matings with *D. simulans* females, there was no difference in the number of sperm transferred by conspecific and heterospecific males, and for single matings with *D. mauritiana* females, heterospecific males (M×S) transferred roughly twice as many sperm as did conspecific males (M×M) or relative to single matings with females of their own species (S×S). The among-cross pattern of sperm transfer by second males was also unexpected. With the exception of the M×SM cross, in which males transferred extremely few sperm, a statistically equivalent number of sperm was transferred by second males in all other heterospecific competition crosses (i.e., S×MS, S×SM, S×MM, and M×MS). The number of sperm transferred in these crosses was roughly one-half that transferred in control rematings (i.e., S×SS and M×MM). These patterns of sperm transfer may be examples of strategic ejaculate tailoring (see the Supplemental Discussion).

Prediction 3 about the extent and asymmetry of sperm displacement among crosses received robust support. The basis for this prediction comes from investigations of *D. melanogaster* using either populations where males or females were experimentally evolved to have longer or shorter sperm or SRs, respectively [29, 30], or for which natural variation in sperm length and other traits was partitioned and fixed

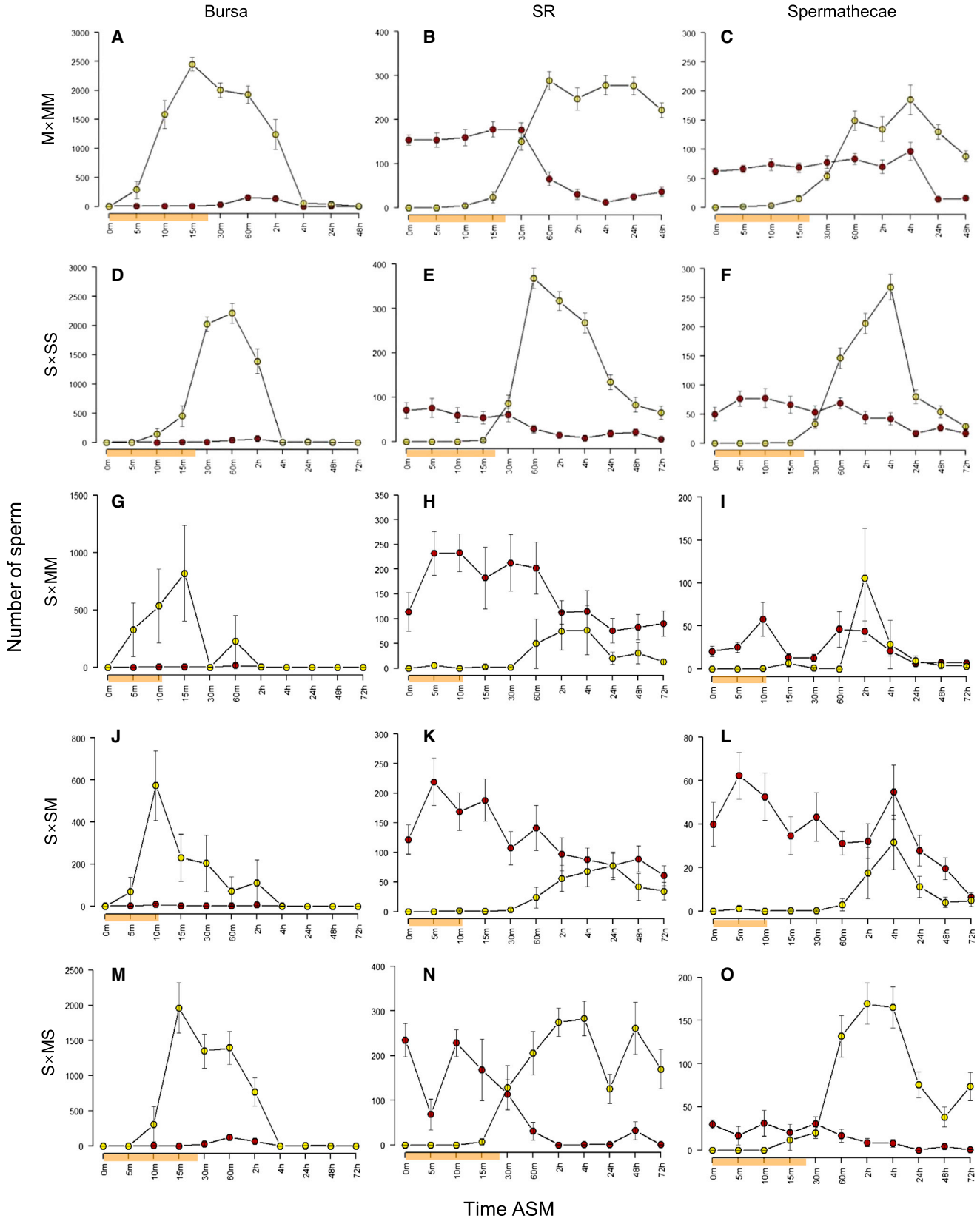

Figure 3. Spatiotemporal Patterns of Sperm Transfer, Storage, and Displacement in the Bursa, SR, and Spermathecae

Mean and SEM for numbers of first-male (red) and second-male (yellow) sperm in the bursa (left column: A, D, G, J, and M), SR (middle column: B, E, H, K, and N), and spermathecae (right column: C, F, I, L, and O) in the M×MM (first row: A–C), S×SS (second row: D–F), S×MM (third row: G–I), S×MS (fourth row: J– L)

(legend continued on next page)

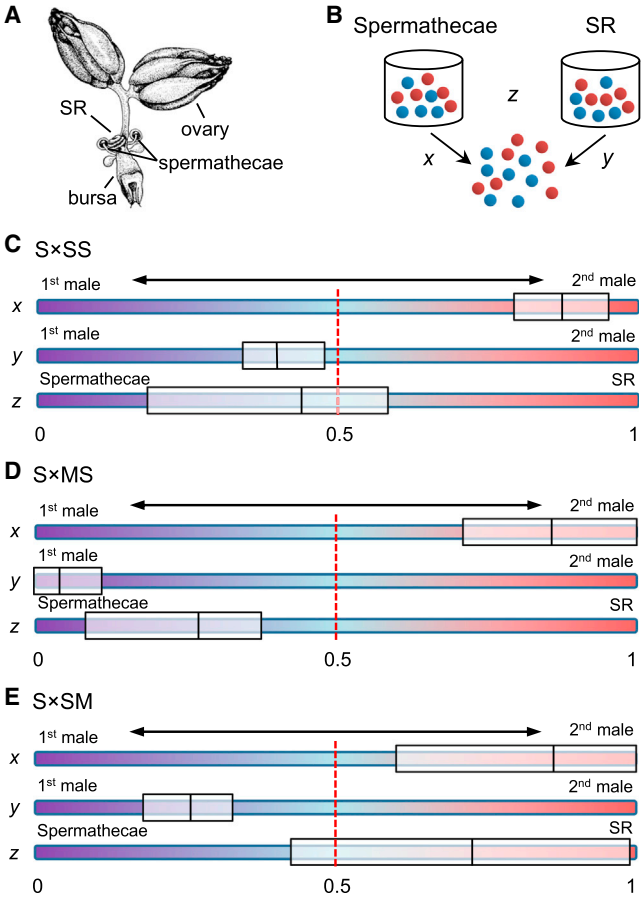

Figure 4. Patterns of Sperm Use Bias Based on Mating Order of the Conspecific and Heterospecific Males

(A) The female reproductive tract of *D. simulans* showing the two types of sperm-storage organ: the seminal receptacle (SR) and the paired spermathecae (reproduced from [27]).

(B) Sources of potential sperm use bias from the spermathecae (x), SR (y), and between the two storage organ types (z) estimated from the analytic model [28].

(C–E) Fertilization bias within the S×SS [27] (C), S×MS (D), and S×SM (E) crosses. Boxes (estimate mean and 95% confidence interval) overlapping with 0.5 indicate no significant fertilization bias. x or $y < 0.5$ indicates a first-male bias, and x or $y > 0.5$ indicates a second-male bias. $z < 0.5$ indicates a bias toward use of sperm from the spermathecae, and $z > 0.5$ indicates a bias toward sperm from the SR.

among isogenic lines [22]. Longer sperm were consistently found to be superior at displacing, and resisting displacement by, shorter sperm [22, 30] (also see [13] for an example in beetles), with the paternity advantage accrued by males producing longer sperm decreasing with decreasing length of the female SR [29]. The observed displacement advantage of *D. simulans* sperm over *D. mauritiana* sperm within *D. simulans* females (Figures 2C and 3 and Table 2) was therefore predicted because the sperm and SRs of *D. simulans* are respectively 12% and 18% longer on average than those of *D. mauritiana* [20]. The lack of this CSP mechanism operating during heterospecific sperm competition in *D. mauritiana* females (i.e., *D. mauritiana* sperm were not superior at displacing *D. simulans* sperm within *D. mauritiana* females) was likewise predicted a priori (Figure 2C and Table 3).

The two remaining mechanisms of CSP that we identified in this study, female sperm ejection and fertilization bias in sperm use [21], present examples of cryptic female choice or female-mediated bias of offspring paternity after mating has occurred [6]. These two mechanisms act at different stages in the post-copulatory process: (1) the formation of the fertilization set and (2) the fertilization of eggs [20, 21]. Sperm ejection has been observed in diverse taxa and experimentally confirmed as a mechanism of cryptic female choice in the domestic fowl (reviewed in [31]). In *Drosophila*, relatively rapid ejection of the ejaculate after remating minimizes the number of second-male sperm that can enter the storage organs and consequently the number of resident, first-male sperm that are displaced from storage. As a consequence, there is a significant negative relationship between time to ejection and P_2 [23]. Prediction 4 was supported, in part, in that *D. simulans* females ejected sperm significantly faster when singly mated with a heterospecific than with a conspecific male (Table 2). For double matings, particularly rapid female ejection was observed only in the S×SM treatment (Figures 2D and 3 and Table 2), which is the cross for which rapid ejection after remating would have the greatest impact biasing fertilization in favor of conspecific sperm. On the other hand, prediction 4 was not supported for crosses with *D. mauritiana* females. Although sample sizes were small, no female either singly mated or remated to a *D. simulans* male (n = 15) ejected sperm during the 5 hr observation period. Work to date with isogenic lines of *D. melanogaster* has found no genetic variation in males for time of sperm ejection by their mates [22] yet substantive genetic variation in females for ejection time [23] (male×female interactions have not yet been examined). The unexpected lack of sperm ejection by *D. mauritiana* females after heterospecific insemination suggests an influence of the ejaculate biochemistry on ejection time (also see Movie S4). It also presents yet another biological justification for prediction 6.

There were not sufficient data to test prediction 5 about *D. mauritiana* females lacking the physiological ability to exert fertilization bias against heterospecific sperm [21]. Nevertheless, the prediction that *D. simulans* females would exert such a bias was strongly supported. The significant shift in the source of sperm for fertilization (z in Figure 4), favoring the spermathecae (which exhibits a second-male fertilization bias; x in Figure 4) in the S×MS cross and the SR (which exhibits a first-male fertilization bias; y in Figure 4) in the S×SM cross represents a sophisticated mechanism to bias paternity in favor of the conspecific sperm irrespective of mating order (i.e., CSP), and one of the clearest examples to date of female sperm choice in any species [32]. It is perhaps noteworthy that females in the S×SM cross exhibited only a moderate, nonsignificant shift in sperm use toward the SR (z in Figure 4E). Females in this cross often (but not always) exhibit exceptionally rapid sperm ejection. If females that employ early ejection as a mechanism of cryptic female choice do not then switch use of sperm-storage organs, the increased variation in the data may explain the trending but nonsignificant result.

and S×SM (fifth row: M–O) for various time points after the start of mating (ASM) are shown. The orange bar represents copulation duration. Copulations with *D. mauritiana* males are much shorter and transfer fewer sperm (G and J) than with *D. simulans* males (B and M). Furthermore, *D. simulans* sperm are much more effective at displacing *D. mauritiana* sperm (N) than *D. mauritiana* sperm are at displacing *D. simulans* sperm (K) or even other *D. mauritiana* sperm (H). (A)–(F) are reproduced from [20].

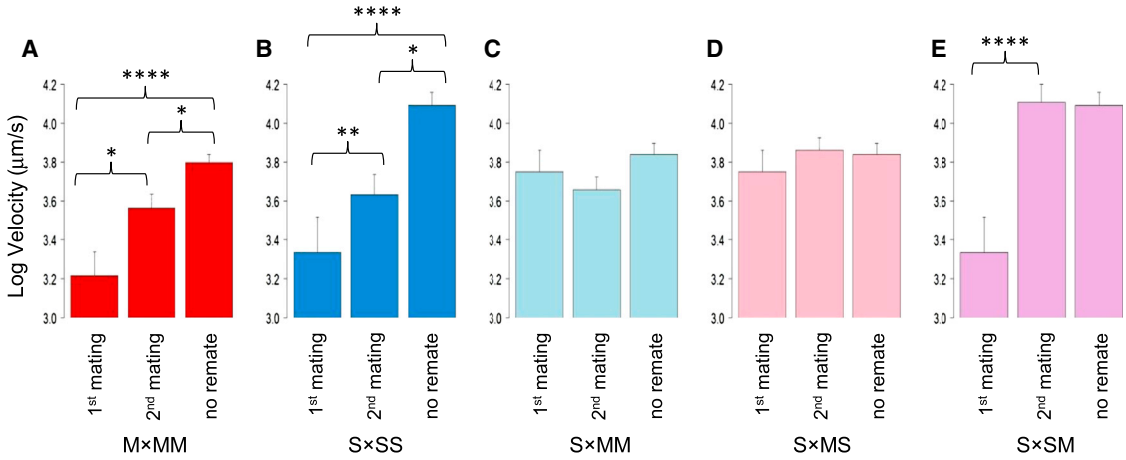

Figure 5. Sperm Velocity Associated with Remating Suggests that Normal Male-Female Interactions Are Disrupted in Hybrid Matings

Mean ± SEM log-transformed velocity (μm/s) of the first male's sperm in the SR measured 60 min after the start of the first mating ("1st mating"), 60 min after the start of the second mating ("2nd mating"), and in females not given the opportunity to remate but dissected on the same timescale as the second mating ("no remate"). Analyses of intraspecific, competitive matings in both *D. mauritiana* (A) and *D. simulans* (B) revealed that sperm swimming velocity increased with storage time (contrast "1st mating" with "no remate"), with the more beneficial, slower swimming speed partially "rescued" by female remating (contrast "2nd mating" with "no remate"). In contrast, the velocity of *D. mauritiana* sperm in a *D. simulans* SR (C and D) did not change over time and resembled at all time points the faster velocity observed after several days in storage in a *D. mauritiana* SR (A, "no remate"), irrespective of the species identity of the second male (C and D). In addition, *D. simulans* sperm velocity was not "rescued" by the female having remated to a *D. mauritiana* male (E, contrast "2nd mating" with "no remate"). All statistical analyses controlled for variation in sperm density within the SR. *p < 0.05, **p < 0.01, ***p < 0.001.

Conversely, females in the S×MS cross, which would not have ejected sperm particularly early after the first, heterospecific mating, showed a strong pattern of sperm use bias (*z* in Figure 4D). These results suggest that the two cryptic female choice mechanisms, ejection and fertilization bias, may be acting in a complementary fashion. Because the sperm-storage organ use plasticity revealed here could not have arisen due to selection to avoid hybridization [25, 26], we postulate that this mechanism of CSP is a consequence of selection for intraspecific cryptic female choice, with heterospecific males representing an extreme form of lower quality male. How the opposing biases between storage organ types arise and are maintained is unknown but may be related to organ-specific patterns of gene expression and specialized secretory activity [33].

Due to strong premating isolation between *D. mauritiana* females and *D. simulans* males [19, 26], it was not possible to test prediction 6 regarding asymmetry of CSP. The single *D. mauritiana* female producing progeny after insemination by both a conspecific and heterospecific male (i.e., M×MS cross), with $P_2 = 0.67$, provides consistent anecdotal support. Asymmetrical reproductive isolation between reciprocal crosses is commonly observed, with hypotheses regarding causative mechanisms (e.g., behavioral, mechanical, and genetic) differing between the stage of isolation (i.e., premating, PMPZ, or postzygotic; e.g., [34]). As illustrated by the present study, knowledge of ejaculate-female interactions provides the basis for particularly precise and testable hypotheses of isolating mechanisms.

Events occurring between copulation and fertilization are multifarious, with myriad male and female traits engaged in genitalic, ejaculate-ejaculate, ejaculate-female, and sperm-egg interactions that potentially contribute to differential competitive fertilization success. These include some of the fastest evolving genes, proteins, and characters known [7, 8]. The few empirical studies of such trait divergence among geographic populations show diversification rates relevant to the speciation process, including coevolving male-female traits demonstrated in the present study to be a "speciation phenotype" (e.g., [35, 36]). We thus predict that mechanisms of gametic isolation and CSP will vary greatly among taxa. Given an allopatric distribution with no secondary contact, PMPZ reproductive isolation played no role in speciation between *D. simulans* and *D. mauritiana*. Rather, the present and previous investigations with this model system [12, 19–21] confirm the potential role of postcopulatory sexual selection in speciation. Application of the high-resolution experimental methods of the present study to other systems, particularly those in earlier stages of speciation, populations occurring in sympatry and subject to reinforcement, will provide valuable insight into the speciation process. Until more studies of PMPZ mechanisms are conducted, the extent and kind of divergence required to generate reproductive isolation will not be known. Some kinds of divergence may quickly result in ejaculate-female incompatibility due, for example, to the inability of sperm to be stored or to survive in storage. In other cases, reproductive processes may be relatively forgiving, with no isolation apparent for single matings and only becoming apparent under the more sensitive assay of sperm competition (i.e., CSP) with the female relatively incompatible with only one of the two competing ejaculates. On the other hand, the reverse pattern is also theoretically possible, given that ejaculates from competing males may interact such that the conspecific seminal plasma may "rescue" the heterospecific sperm [22, 37].

Experimental Procedures

For a detailed description of all procedures, see the Supplemental Experimental Procedures.

Transgenic *D. simulans* and *D. mauritiana* with GFP- or RFP-labeled sperm heads enabled the unambiguous discrimination of each males' sperm in double-mated females (Movies S1, S2, and S3) to quantify in vivo sperm velocity, numbers of competing sperm in all regions of the female's lower reproductive tract (i.e., bursa, SR, spermathecae; Figure 3)

Table 3. Patterns Mechanisms of Postmating, Prezygotic Reproductive Isolation Are Different when Sperm Compete within *Drosophila mauritiana* Females

	M×M[a]	M×MM[a]	M×S	M×MS	M×SM
Mating rate	0.81 (800)	0.40 (589)	0.05 (127)	0.016 (816)	NA
Mating success	0.95 (55)	0.98 (109)	1.0 (6)	0.93 (14)	1.0 (4)
Copulation duration (min)	15.8 ± 0.24 (234)	23.7 ± 0.69 (141)	22.0 ± 0.93 (6)	18.2 ± 1.80 (11)	19.5 ± 3.88 (4)
Sperm transferred	1532 ± 103 (22)	2348 ± 93 (61)	2758 ± 242 (6)	1443 ± 380 (9)	276 ± 152 (4)
Sperm stored (total)	430 ± 17 (30)	545 ± 21 (61)	687 ± 41 (6)	325 ± 91 (9)	237 ± 123 (4)
Sperm stored (second male)	NA	425 ± 23 (61)	NA	309 ± 94.8 (9)	165 ± 128 (4)
Residual sperm at remating	NA	252 ± 9 (160)	NA	62.1 ± 21.6 (9)	76.5 ± 38.5 (4)
Ejection rate	1.0 (51)	0.98 (17)	0 (6)	0 (9)	0 (5)
Ejection time (min ASM)	125 ± 4.5 (45)	101 ± 6.0 (15)	NA	NA	NA
Proportion of sperm displaced	NA	0.48 ± 0.04 (57)	NA	0.56 ± 0.17 (9)	0.09 ± 0.06 (4)

Trait means ± SEM and sample sizes for reproductive and sperm traits for first (M×M) and second matings of *D. mauritiana* (M×MM), and first (M×S) and second (M×MS and M×SM) hybrid matings.
[a]Data are from [20].

and paternity. We used single matings of *D. simulans* (S) females with *D. mauritiana* (M) males (S×M) and double matings of *D. simulans* females to two *D. mauritiana* males (S×MM), a *D. simulans* male followed by a *D. mauritiana* male (S×SM), or the reciprocal cross (S×MS) to quantify sperm transfer, storage, and use and the dynamics of sperm competition. Crosses were similarly conducted using *D. mauritiana* females (M×S, M×MS, and M×SM). For each cross, we quantified (1) numbers of sperm transferred, (2) numbers of sperm stored in each type of sperm storage organ, (3) proportion of resident (first-male) sperm displaced from storage, (4) timing of ejection of excess second-male and displaced first-male sperm, (5) velocity of first-male sperm in the SR, (6) fertilization bias toward the first or second male within each of the two types of storage organ and sperm use bias between storage organ types, and (7) paternity success over 72 hr after remating. Data are compared among crosses conducted here and with intraspecific crosses (M×MM and S×SS) using identical methods [20]. Unless otherwise stated, all data refer to events associated with the second mating. Statistical analyses were performed in R version 2.12.1 [38].

Accession Numbers

All original data have been deposited in the Dryad Repository at http://dx.doi.org/10.5061/dryad.32665.

Supplemental Information

Supplemental Information includes Supplemental Discussion, Supplemental Experimental Procedures, and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.07.086.

Acknowledgments

We thank E. Droge-Young, D. Higginson, L. Paish, and A. Rao for assistance in data collection and P. E. Deane, E. Droge-Young, B. Gress, E.L. Larson, R. Montgomerie, F.A. Ransler, J.A.C. Uy, and two anonymous referees for helpful comments on the manuscript. This work was funded by the NSF (DEB-0814732, DEB-1021240, and DEB-1145965 to S.P., J.M.B., S.L., and M.K.M.), the Swiss NSF (fellowships PBSKP3_130878 and PA00P3_134191 to S.L.), and the Academy of Finland (grant 135684 to O.A.-H.).

Received: June 22, 2013
Revised: July 31, 2013
Accepted: July 31, 2013
Published: September 26, 2013

References

1. Butlin, R., Debelle, A., Kerth, C., Snook, R.R., Beukeboom, L.W., Castillo Cajas, R.F., Diao, W., Maan, M.E., Paolucci, S., Weissing, F.J., et al.; Marie Curie SPECIATION Network. (2012). What do we need to know about speciation? Trends Ecol. Evol. *27*, 27–39.
2. Coyne, J.A., and Orr, H.A. (2004). Speciation (Sunderland: Sinauer).
3. Ritchie, M.G. (2007). Sexual selection and speciation. Annu. Rev. Ecol. Evol. Syst. *38*, 79–102.
4. Kraaijeveld, K., Kraaijeveld-Smit, F.J.L., and Maan, M.E. (2011). Sexual selection and speciation: the comparative evidence revisited. Biol. Rev. Camb. Philos. Soc. *86*, 367–377.
5. Howard, D.J., Palumbi, S.R., Birge, L., and Manier, M.K. (2009). Sperm and speciation. In Sperm Biology: An Evolutionary Perspective, T.R. Birkhead, D.J. Hosken, and S. Pitnick, eds. (London: Academic Press), pp. 367–403.
6. Eberhard, W.G. (1996). Female Control: Sexual Selection by Cryptic Female Choice (Princeton: Princeton University Press).
7. Ravi Ram, K., and Wolfner, M.F. (2007). Seminal influences: *Drosophila* Acps and the molecular interplay between males and females during reproduction. Integr. Comp. Biol. *47*, 427–445.
8. Pitnick, S., Wolfner, M.F., and Suarez, S.S. (2009). Ejaculate-female and sperm-female interactions. In Sperm Biology: An Evolutionary Perspective, T.R. Birkhead, D.J. Hosken, and S. Pitnick, eds. (London: Academic Press), pp. 247–304.
9. Higginson, D.M., Miller, K.B., Segraves, K.A., and Pitnick, S. (2012). Female reproductive tract form drives the evolution of complex sperm morphology. Proc. Natl. Acad. Sci. USA *109*, 4538–4543.
10. Howard, D.J. (1999). Conspecific sperm and pollen precedence and speciation. Annu. Rev. Ecol. Syst. *30*, 109–132.
11. Gregory, P.G., and Howard, D.J. (1994). A postinsemination barrier to fertilization isolates two closely related ground crickets. Evolution *48*, 705–710.
12. Price, C.S.C., Kim, C.H., Posluszny, J., and Coyne, J.A. (2000). Mechanisms of conspecific sperm precedence in *Drosophila*. Evolution *54*, 2028–2037.
13. Rugman-Jones, P.F., and Eady, P.E. (2007). Conspecific sperm precedence in *Callosobruchus subinnotatus* (Coleoptera: Bruchidae): mechanisms and consequences. Proc. Biol. Sci. *274*, 983–988.
14. Tyler, F., Harrison, X.A., Bretman, A., Veen, T., Rodríguez-Muñoz, R., and Tregenza, T. (2013). Multiple post-mating barriers to hybridization in field crickets. Mol. Ecol. *22*, 1640–1649. http://dx.doi.org/10.1111/mec.12187.
15. Shaw, K.L., and Mullen, S.P. (2011). Genes versus phenotypes in the study of speciation. Genetica *139*, 649–661.
16. Uy, J.A.C., Moyle, R.G., Filardi, C.E., and Cheviron, Z.A. (2009). Difference in plumage color used in species recognition between incipient species is linked to a single amino acid substitution in the melanocortin-1 receptor. Am. Nat. *174*, 244–254.
17. Oh, K.P., Fergus, D.J., Grace, J.L., and Shaw, K.L. (2012). Interspecific genetics of speciation phenotypes: song and preference coevolution in Hawaiian crickets. J. Evol. Biol. *25*, 1500–1512.
18. McDermott, S.R., and Kliman, R.M. (2008). Estimation of isolation times of the island species in the *Drosophila simulans* complex from multilocus DNA sequence data. PLoS ONE *3*, e2442.
19. Price, C.S.C. (1997). Conspecific sperm precedence in *Drosophila*. Nature *388*, 663–666.
20. Manier, M.K., Belote, J.M., Berben, K.S., Lüpold, S., Ala-Honkola, O., Collins, W.F., and Pitnick, S. (2013). Rapid diversification of sperm precedence traits and processes among three sibling Drosophila species. Evolution *67*, 2348–2362.

21. Manier, M.K., Lüpold, S., Pitnick, S., and Starmer, W.T. (2013). An analytical framework for estimating fertilization bias from multiple sperm-storage organs during sperm competition. Am. Nat. *182*, 552–561.
22. Lüpold, S., Manier, M.K., Berben, K.S., Smith, K.J., Daley, B.D., Buckley, S.H., Belote, J.M., and Pitnick, S. (2012). How multivariate ejaculate traits determine competitive fertilization success in *Drosophila melanogaster*. Curr. Biol. *22*, 1667–1672.
23. Lüpold, S., Pitnick, S., Berben, K.S., Blengini, C.S., Belote, J.M., and Manier, M.K. (2013). Female mediation of competitive fertilization success in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *110*, 10693–10698.
24. Manier, M.K., Belote, J.M., Berben, K.S., Novikov, D., Stuart, W.T., and Pitnick, S. (2010). Resolving mechanisms of competitive fertilization success in *Drosophila melanogaster*. Science *328*, 354–357.
25. Lorch, P.D., and Servedio, M.R. (2007). The evolution of conspecific gamete precedence and its effect on reinforcement. J. Evol. Biol. *20*, 937–949.
26. Coyne, J.A. (1989). Genetics of sexual isolation between two sibling species, *Drosophila simulans* and *Drosophila mauritiana*. Proc. Natl. Acad. Sci. USA *86*, 5464–5468.
27. Patterson, J.T. (1943). Studies in the genetics of *Drosophila*. III. The Drosophilidae of the Southwest. University of Texas Publications *4313*, 7–216.
28. Bjork, A., Starmer, W.T., Higginson, D.M., Rhodes, C.J., and Pitnick, S. (2007). Complex interactions with females and rival males limit the evolution of sperm offence and defence. Proc. Biol. Sci. *274*, 1779–1788.
29. Miller, G.T., and Pitnick, S. (2002). Sperm-female coevolution in *Drosophila*. Science *298*, 1230–1233.
30. Pattarini, J.M., Starmer, W.T., Bjork, A., and Pitnick, S. (2006). Mechanisms underlying the sperm quality advantage in *Drosophila melanogaster*. Evolution *60*, 2064–2080.
31. Dean, R., Nakagawa, S., and Pizzari, T. (2011). The risk and intensity of sperm ejection in female birds. Am. Nat. *178*, 343–354.
32. Birkhead, T.R. (1998). Cryptic female choice: criteria for establishing female sperm choice. Evolution *52*, 1212–1218.
33. Prokupek, A.M., Kachman, S.D., Ladunga, I., and Harshman, L.G. (2009). Transcriptional profiling of the sperm storage organs of *Drosophila melanogaster*. Insect Mol. Biol. *18*, 465–475.
34. Turelli, M., and Moyle, L.C. (2007). Asymmetric postmating isolation: Darwin's corollary to Haldane's rule. Genetics *176*, 1059–1088.
35. Pitnick, S., Miller, G.T., Schneider, K., and Markow, T.A. (2003). Ejaculate-female coevolution in *Drosophila mojavensis*. Proc. Biol. Sci. *270*, 1507–1512.
36. Fricke, C., and Arnqvist, G. (2004). Divergence in replicated phylogenies: the evolution of partial post-mating prezygotic isolation in bean weevils. J. Evol. Biol. *17*, 1345–1354.
37. Hodgson, D.J., and Hosken, D.J. (2006). Sperm competition promotes the exploitation of rival ejaculates. J. Theor. Biol. *243*, 230–234.
38. R Development Core Team. (2011). R: A Language and Environment for Statistical Computing (Vienna: R Foundation for Statistical Computing).